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Characterisation and properties of ectosomes released by human polymorphonuclear neutrophils

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Abstract

Human neutrophils release vesicles when activated in vitro and in vivo, in local and systemic inflammation. We have suggested that the presence of these vesicles is due to ectocytosis, defined as the release of rightside-out oriented vesicles expressing a select set of membrane proteins. Herein we have characterised the vesicles released by neutrophils to be ectosomes with specific properties. They contained cytosolic F-actin indicating their outside-out orientation. They bound Annexin V, suggesting that they expose phosphatidylserine, similarly to platelet microparticles. They expressed a subset of cell surface proteins (selectins and integrins, complement regulators, HLA-1, FcγRIII, and CD66b, but not CD14, FcγRII, and CD87). There was no specificity for transmembrane or glycosyl-phosphatidylinositol-linked proteins and, unexpectedly, L-selectin, known to be cleaved from the surface of activated neutrophils, was present. Ectosomes exposed active enzymes released by neutrophils upon degranulation (matrix metalloproteinase-9, myeloperoxidase, proteinase 3, and elastase). In particular, released myeloperoxidase was able to bind back to ectosomes. The purified complement protein C1q and C1q from serum bound to ectosomes as well. Another aspect of ectosomes was that they became specifically adherent to monocytic and endothelial cells. These observations suggest that neutrophil-derived ectosomes have unique characteristics that make them candidates for playing roles in inflammation and cell signaling.

Introduction

Many eukaryotic cells release vesicles spontaneously or under appropriate stimulation. Exosomes are preformed membrane vesicles, which are stored in cellular compartments named multivesicular bodies, and secreted when the multivesicular bodies fuse with the cell membrane [1]. Many haematopoietic cells, including reticulocytes, platelets, and leucocytes secrete exosomes. For reticulocytes, exosomes mediate the clearance of obsolete proteins such as the transferrin receptor. Those released by B-lymphocytes bind firmly to follicular dendritic cells and may have the function to present antigens to T lymphocytes [2]. Exosomes of cytotoxic T lymphocytes may efficiently deliver cytolytic substances to specific targets [3]. The structure of such exosomes has now been characterised [4].

Beside the release of preformed vesicles, many cells shed small membrane vesicles which are formed directly from the cell membrane [5]. This reaction provides, for instance, protection against complement attack, because it allows the removal of the C5b-9 attack complex from the cell surface as shown for polymorphonuclear leucocytes (PMNs), oligodendrocytes, and even erythrocytes [6–8]. Stein and Luzio coined the term ectocytosis for the release of rightside-out oriented vesicles (ectosomes) from the surface of PMNs attacked by complement [9]. However, ectocytosis did not only correspond to the removal of the C5b-9 complex, but also to a specific sorting of membrane proteins into the shed ectosomes. Enrichment in cholesterol and diacylglycerol in the ectosome membrane attested for a specific sorting of lipids as well. Hess et al. have provided evidence that when

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PMNs are stimulated in vitro with formyl-methionyl-leucyl-phenylalanine (fMLP) or exposed to calcium ionophores, they release small vesicles in minutes which have many of the properties expected for ectosomes due to complement attack [10]. They expressed a specific set of membrane proteins, including the complement receptor 1 (CR1), and in addition, proteases such as myeloperoxidase (MPO) and elastase, suggesting that they may function as extracellular antibacterial organelles. Interestingly, similar vesicles were isolated from synovial fluids of patients with arthritis, bronchoalveolar lavage fluids in bacterial pneumonia, and skin blisters induced by Cantharidin, all situations in which PMNs are predominant in the exsudate. In addition, electron-microscopy images showed the formation of such vesicles on PMNs by ectocytosis minutes after the stimulatory agent was added.

Various names have been used to describe vesicles released by cells including microparticles, particles, microvesicles, and vesicles, probably because the mechanism involved in their release was not always studied in detail [11–21]. A major fraction of microparticles shed by activated platelets corresponds certainly to the definition of ectosomes, as do many others released by monocytes, tumor cells, and fibroblasts [13,20,22,23]. Various functions may be mediated by ectosomes. Mononuclear cell-released microparticles transfer the chemokine receptor CCR5 to cells not expressing it, enabling HIV to infect the recipient cell [15]. The rapid secretion of IL-1 β by THP-1 cells is due to the shedding of ectosomes loaded with IL-1β [14]. Platelet- and monocyte-derived ectosomes induce the coagulation cascade and inflammatory processes by activating endothelial cells [11,20,24]. Microparticles released by leucocytes—mainly PMNs—may activate endothelial cells as well [18]. Such microparticles were released by resting leucocytes, but the addition of a stimulus like fMLP increased their shedding manyfold. Although the nature of the microparticles was not further analysed, one might speculate that they were ectosomes.

Because vesicles released by activated PMNs may have many biological activities, we set out to further analyse their structure/function characteristics. The data obtained here provide evidence that they are rightside-out oriented, express phosphatidyl-serine, have a very unique pattern of in- and out-shuffled cell-membrane proteins, conserve L-selectin which in contrast is cleaved from the surface of activated PMNs, acquire a specific panel of proteolytic enzymes, bind C1q, and can target endothelial as well as monocytic cells.

Material and methods

Antibodies

All antibodies used were monoclonal mouse anti-human antibodies (mAb). The following mAb were used: phycoerythrin coupled mAb directed against CD79 and L-selectin/CD62L (Pharmingen/Becton Dickinson), purified mAb against LFA-1/CD11a, Mac-1/CD11b (Pharmingen), CD66b, FcγRIII/CD16 (Immunotech, Marseille, France), myeloperoxidase (MPO), human leucocyte antigen-I (HLA-I; Dako, Glostrup, Denmark), MCP/CD46, CD59 (Sanbio, Netherlands), proteinase 3 (PR3) (CLB, Amsterdam, Netherlands), matrix metalloproteinase-9 (MMP-9) (R&D Systems, Minneapolis, MN, USA), urokinase plasminogen activator receptor (uPAR)/CD87 (Diaclone research, Besançon, France), DAF/CD55 (Biosource, Camarillo, CA), FcγRII/CD32 (NeoMarkers, Fremont, CA, USA), C1q (Quidel, San Diego, CA, USA), FLAG-peptide (IgG1 control directed against the octa-peptide N-Asp Tyr Lys Asp Asp Asp Asp Lys-C) (Eastman Kodak Company, New Haven, CT, USA), and CR1/CD35 (clone 3D9). All nonlabeled mAb were biotinylated prior to use (see below).

Biotinylation of monoclonal antibodies

Ab were incubated for 1 h at 22°C in the presence of 1 μL biotin-ester (10 mg/mL in DMSO)/50 μg of antibody in sodium borate buffer (0.1 M, pH 8.8), final volume 2 mL. Biotinylated mAb were dialyzed against phosphate buffered saline (PBS) overnight at 4°C in a Slide-A-Lyzer 10,000 MW cut-off dialysis cassette (Pierce, Rockford, IL, USA). Biotinylated mAb were recovered and concentrated with microsep centrifugal concentrators (10,000 MW cut-off, Pall Filtron Corp.) to a final mAb-concentration of 250 μg/mL. Biotinylated mAb were stored at 4°C.

Isolation and stimulation of PMNs

PMNs were isolated from fresh buffy coats of normal donors according to the technique described previously [10]. Briefly, a fresh buffy coat obtained from approximately 400 mL of normal donor blood was diluted 1/1 (v/v) with PBS-ethylenediaminetetraacetic acid (EDTA) (2 mM), mixed gently with 0.25 vol of 4% Dextran T500 (Amersham Pharmacia Biotech, Dübendorf, Switzerland), and left for 30 min for erythrocyte sedimentation. The leucocyte-rich supernatant (S/N) was aspirated and centrifuged for 10 min at 200*g*. The pellet was resuspended for 1 min in 9 mL of ultrapure water to lyse erythrocytes. Isotonicity was restored by addition of 3 mL of KCl (0.6 M) and 40 mL of NaCl (0.15 M). Cells were then centrifuged 10 min at 350*g*, and resuspended in 20 mL of PBS-EDTA (2 mM). This suspension was layered over 20 mL of Ficoll-Hypaque (Sigma, St. Louis, MO, USA) and centrifuged for 30 min at 350*g*. The PMN-rich pellet was recovered and washed twice in PBS-EDTA (2 mM). All manipulations were performed at 4°C, thus minimizing PMN activation.

For stimulation, PMNs (10^7 cells/mL) were diluted 1/1 (v/v) in prewarmed (37°C) RPMI 1640 (Life Technologies, Basel, Switzerland) with fMLP, ionomycin, or PMA [1 μM, 5 μM, 10 nM final concentrations respectively, all from Sigma (St. Louis, MO, USA)], and incubated for 20 min at

37°C in a water bath. Cell activation was stopped by putting the cell suspensions on ice, and PMNs were removed by centrifugation (two consecutive runs, 20 min each, 4000*g* at 4°C). The supernatant (S/N) was concentrated ~50-fold with Centriprep centrifugal filter devices (10,000 MW cut-off, Millipore Corporation, Bedford, MA, USA) and stored in aliquots at −80°C until use.

Transmission electron microscopy of ectosomes/negative staining

After ultracentrifugation, ectosomes were resuspended in PBS and then fixed in 1% glutaraldehyd (final concentration) for 20 min at room temperature. Ectosomes were then adsorbed to parlodion-coated copper grids. After washing, samples were stained with 2% uranylacetate before being observed in a Philips Morgani 268 D transmission electron microscope operated at 80 kV.

Fluorescence activated cell scanning (FACScan)-analysis of ectosomes

Samples were analysed with a FACScan flow cytometer from Becton-Dickinson (Mountain View, CA, USA). The light scatter and fluorescence channels were set at logarithmic gain. An acquisition threshold was set on the forward scatter in order to reduce background-signal. In order to analyse ectosomes by FACScan, concentrated PMN-S/N (see above) was ultracentrifuged for 30 min at 160,000*g*/4°C in a SW55ti rotor (Beckman Instruments Inc., Palo Alto, CA, USA). Pelleted ectosomes were washed twice with 0.9% NaCl (B. Braun Medical AG, Emmenbrücke, Switzerland) and incubated at 22°C for 10 min in 100 μL 0.9% NaCl/10% normal mouse serum (ultracentrifuged prior to use in order to remove aggregates) (Cedarlane laboratories Ltd., Hornby, Canada). Blocked ectosomes were labeled with 5 μg/mL of biotinylated mAb, or a 1/200 dilution of fluorochrome labeled mAb, in FACScan buffer (PBS, 1% bovine serum albumin (BSA), 10 mM sodium azide) for 10 min at 22°C. Ectosomes were then pelleted by ultracentrifugation (30 min, 160,000*g*/4°C), and washed twice in FACScan buffer. Bound biotinylated antibodies were revealed with 2 μL streptavidin-phyco-erythrin/100 μL FACScan buffer (incubated for 10 min at 22°C). Labeled ectosomes were then resuspended in 200 μL 0.9% NaCl and analysed on a FACScan. Forward (FSC) and side scatter (SSC) as well as fluorescence channels (FL1 and FL2) were set on logarithmic scales.

Membrane labeling of ectosomes

An amphiphilic cell linker dye kit (PKH67, Sigma, St. Louis, MO, USA) was used, following the labeling procedure provided by the manufacturer. Briefly, ectosomes resuspended in 200 μL Diluent C were incubated with 200 μL diluent C/dye solution (dye diluted 1/200) for 30 s at 22°C, with gentle shaking. One milliliter RPMI 1640 (without phenol red) was added to stop the reaction. Labeled ectosomes were separated from the remaining unbound dye by ultracentrifugation (45 min, 160,000*g*/4°C) and washed with 0.9% NaCl. In order to avoid capping of Ab with dye, membrane labeling was made prior to antibody-binding steps.

Binding of annexin V to ectosomes

Ectosomes were isolated from concentrated PMN-S/N and washed as described above. Washed ectosomes were resuspended in either 100 μL 0.9% NaCl, or AnV binding buffer (10 mM Hepes/NaOH, 140 mM NaCl, pH 7.4) with or without 2.5 mM $CaCl_2$. Two microliters AnV-FITC or AnV-biotin was added to each reaction. After incubation for 10 min/22°C, ectosomes were washed twice in their respective buffer. When using AnV-biotin, a second step with SA-PE was performed as described above. Ectosomes were then resuspended in 200 μL 0.9% NaCl and analysed by FACScan.

Western blot analysis/zymography of matrix metalloproteinase-9

Western blotting and zymography were performed after separation of ectosomal proteins by 7.5% sodium-dodecylsulfate polyacrylamide-gel-electrophoresis (SDS-PAGE). For zymography, gels were supplemented with gelatin (1 mg/mL) (Sigma, St. Louis, MO, USA). For zymography, gels were incubated in zymography buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$) overnight at 37°C and stained in Brilliant blue. Recombinant active MMP-9 was used as positive control (Oncogene, Cambridge, MA, USA; 0.5 μg/lane). For Western blots, gels were transferred onto nitrocellulose membranes, which were blocked overnight at 4°C in PBS/6% skimmed milk powder. Membranes were then washed briefly in PBS/0.01% Tween 20 and incubated with anti-MMP-9 antibodies (0.5 μg/mL in PBS/1% BSA) for 1 h at 22°C. Membranes were then washed three times for 10 min in PBS/0.01% Tween 20 and bound mAb detected using goat anti-mouse antibodies. Finally, membranes were incubated for 45 min in horseradish-peroxidase coupled streptavidin diluted in PBS-BSA. Detection was by enhanced chemiluminescence (ECL kit, Amersham Pharmacia Biotech) with BioMax films (Kodak; Rochester, NY, USA).

Alternatively, to check for the presence of MMP-9 inside ectosomes, we probed whole ectosomes as well as sonicated ectosomes with an EnzChek Gelatinase/Collagenase Assay kit (Molecular Probes, Eugene, OR, USA), following the manufacturer's indications.

MPO and elastase activity assays

Enzymatic activity of MPO was determined in a colorimetric assay using whole ectosomes, where 50 μL of orthophenyldiamine solution (2 μg in 5 mL citrate-phosphate buffer, pH 5, with 2 μL of 35% H_2O_2) was incubated with 100 μL of the sample to be tested. The reaction was stopped using 2.5 M H_2SO_4, and the absorbance measured with a microplate reader (Thermo Max, Molecular Devices, Menlo Park, CA, USA) at 490 nm [25]. Enzymatic activity of elastase was analysed using an EnzChek Elastase Assay kit (Molecular Probes, Eugene, OR, USA). Where indicated, elafin (a specific inhibitor of elastase, kindly provided by Dr. Dougie Paterson, Zeneca Pharmaceuticals, GB) was used at a concentration of 30 μg/mL. To check for the presence of elastase inside ectosomes we assessed elastase activities of whole ectosomes as well as sonicated ectosomes with this kit.

Detection of ectosomes in whole blood

Fresh citrate anti-coagulated blood was obtained from normal blood donors. For activation, 5 mL of whole blood was incubated with 1 μM fMLP for 20 min at 37°C. Immediately after activation, cells were removed by centrifugation (10 min, 1000*g*, 25°C) and S/N stored at −80°C until use.

For isolation of blood-borne vesicles, including ectosomes, plasma from activated whole blood was ultracentrifuged as for PMN-S/N. Vesicles were then stained with AnV and mAb against CD66b. Fluorescence of vesicles was detected by FACScan-analysis.

Binding of exogenous MPO to MPO-deficient ectosomes

MPO-deficient as well as normal PMNs were isolated and activated as described above. Ectosome-free normal PMN-S/N was kept and stored until further use at 4°C. After a washing step with 0.9% NaCl, MPO-deficient ectosomes were incubated 10 min at 22°C with 200 μL of ectosome-free normal PMN-S/N, 100 ng of purified MPO added to 200 μL PBS, or 200 μL PBS. Ectosomes from normal donors were incubated with PBS only. Ectosomes were then washed twice and stained for MPO with biotinylated mAb anti-human MPO, followed by streptavidin-phyco-erythrin as described above. Labeled ectosomes were analysed by FACScan-analysis.

Binding of C1q to ectosomes

Ectosomes were isolated as described above. Ectosomes were then ultracentrifuged and incubated, after gentle resuspension, for 30 min at 4°C in 100 μL of 0.9% NaCl alone or 0.9% NaCl supplemented with either 1 μg of purified C1q (Calbiochem, La Jolla, CA, USA), normal human serum (1/10 final dilution) or heat-inactivated normal human serum (1/10 final dilution; heat inactivation: 30 min at 56°C). The binding of C1q was then detected using a biotinylated anti-C1q antibody and streptavidin-phyco-erythrin, as described for all other FACScan-analyses of ectosomes.

Cell cultures

All cultures were maintained in 5% CO_2 at 37°C and medium replaced every second day. Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (San Diego, CA, USA) and cultured in medium consisting of M199 supplemented with 20% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 100 μg/mL heparin, and 50 μg/mL endothelial cell growth supplement (Sigma, St. Louis, MO, USA). Cells were split by trypsinization (0.025% trypsin solution) when confluent. THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine.

Binding assays of ectosomes to human erythrocytes, and endothelial and phagocytic cells

Confocal microscopy

Ectosomes were prepared and labeled as described above. HUVEC (of the second to fifth passage) were cultured on gelatin-coated culture slides. THP-1 cells were cultured on uncoated culture slides for 3 days in the presence of 160 nM PMA for differentiation into phagocytic cells. HUVEC and THP-1 cells were then incubated with labeled ectosomes for 30 min, washed three times with medium, and analysed (Zeiss microscope). For these experiments, ectosomes were counted by FACS analyses, gating on CD66b positive events. Incubations were performed using a cell to ectosome ratio of 1:10.

FACScan analysis

Erythrocytes were isolated from freshly drawn EDTA-anticoagulated blood from a normal donor by dextran sedimentation. Labeled ectosomes were incubated for 30 min at 37°C with the different cell types. Binding assays were performed using 10^6 erythrocytes/mL in a final volume of 100 μL. The same concentration was used for HUVEC and THP-1 cells. For blocking experiments, prior to adding labeled ectosomes, cells were incubated for 10 min with the indicated proportions of unlabeled ectosomes. Cells were then washed, resuspended, and analysed by FACScan. As mentioned above, incubations were performed using a cell to ectosome ratio of 1:10.

Two-dimensional gel electrophoresis and data analysis

A commercial sigmoidal immobilised pH gradient (IPG) going from pH 3.5 to 10.0 was used for first-dimensional separation. After equilibration, IPG gel strips were trans-

ferred for the second dimension onto vertical gradient slab gels (9–16% T, 2.6% C) and run with the Laemmli-SDS-discontinuous system. Protein detection was done using a sensitive ammoniacal silver stain or Coomassie brilliant blue R-250 (0.1% w/v) and methanol (50% v/v) for 30 min. Destaining was done in a solution containing methanol (40% v/v) and acetic acid (10% v/v). Gels were then scanned using a laser densitometer (APB, Uppsalla, Sweden). The 2-D PAGE image computer analysis was carried out using the MELANIE 3 software package (GeneBio, Geneva, Switzerland). Optical density, area, and volume were computed and related to protein concentration. Also, the relative optical density and relative volume was calculated in order to correct for differences in gel staining. Differentially expressed proteins were determined by differential analysis and significance in differences was tested using Student's t test. A $P < 0.05$ was considered significant.

Protein identification by tandem mass spectrometry (MS)

Coomassie blue 2-DE spots were excised from the 2-D gel and destained with 100 μL of 30% acetonitrile in 50 mM ammonium bicarbonate at 37°C for 45 min. The S/N was discarded and the gel spots dried in a SpeedVac for 30 min. The gel spots were rehydrated with 10 μL of a solution containing 6.25 ng/μL of porcine trypsin in 50 mM ammonium bicarbonate buffer for 45 min on ice. Digestion was carried out overnight at 37°C. Resulting peptides were extracted at 22°C by two sequential extraction steps of 20 min. The first extraction is performed with 20 μL of 1% TFA and the second one with 20 μL of 0.1% TFA in 50% acetonitrile. The combined extracts were concentrated in a SpeedVac to approximately 2 μL, redissolved in 30–40 μL of 0.1% TFA, and dried again down to approximately 2 μL. Then 5 μL of a 0.1% HCOOH solution was added. Peptide extract (5 μL) was loaded onto a 75 μm diameter and 10 cm long C18 microcolumn. Peptides were eluted at 400 nL/min by a linear gradient of acetonitrile in 0.1% HCOOH and directly analysed by nanoESI-MS/MS conducted on a Q-TOF mass spectrometer from Micromass (Manchester, UK). MS/MS spectra were acquired by automatic switching between MS and MS/MS mode by the instrument. Acquired fragment ion spectra were searched by Mascot against databases such as SWISS-PROT, TrEmbl, or ESTs databases.

Results

Qualitative analysis of ectosome preparations

Ectosome preparations were tested for their quality and purity by electron microscopy. As shown in Fig. 1a, ectosome preparations did not present any major impurities. Fig. 1b, c, and d represent higher magnification pictures of ectosomes, showing again the absence of any microparticles other than ectosomes in our preparations. The size heterogeneity of ectosomes is documented in Fig. 1b, which shows ectosomes of different sizes, located next to each other (arrows).

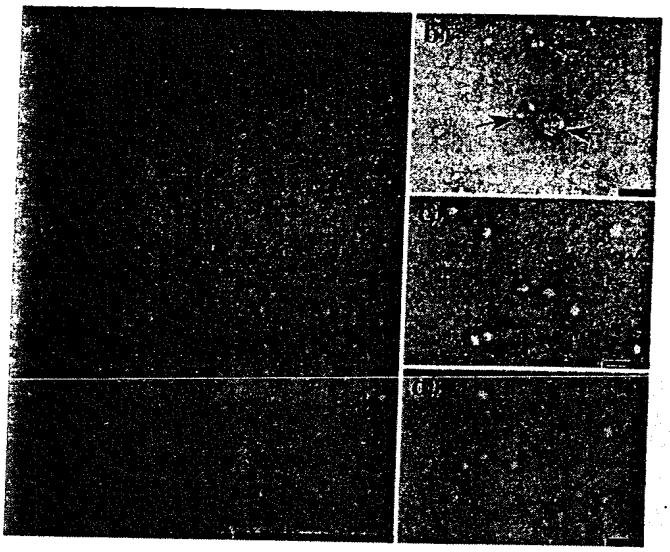


Fig. 1. Electron microscopy of ectosomes, (a) Overview of the purity of ectosome preparations used for analyses. Size bar: 500 nm. (b), (c) and (d) Higher magnification picture of ectosomes. All size bars: 100 nm.

Flow-cytometry-based detection of PMN-derived vesicles

The vesicles released by PMNs activated with 1 μM fMLP were recovered after 20 min of incubation at 37°C by differential centrifugation. First, PMNs were pelleted by centrifugation at 4000g for 2X20 min, and the supernatant containing the vesicles was then ultracentrifuged at 160,000g for 30 min. The pelleted vesicles were resuspended and could be dissociated by gentle mixing in 0.9% NaCl. By flow cytometry such vesicles were found to form a distinct FSC/SSC-population. The size heterogeneity was reflected by a simultaneous scattering toward higher FSC and SSC values. A typical FSC/SSC plot of purified vesicles and the standard gate as set for their analysis is shown in Fig. 2A. The detection of synthetic fluorescent beads of 200 nm diameter within the gate set for vesicles confirmed their approximate size (of range 50 to 200 nm) [10] (data not shown).

Using an amphiphilic, membrane-interchelating dye, particles within the above-defined gate uniformly acquired fluorescence (Fig. 2B). Interestingly the intensity of the fluorescence correlated directly with both FSC and SSC, indicating that larger vesicles had taken up more dye (data not shown).

Under many circumstances, vesicles released by activated cells express phosphatidylserine (PS), a marker for the loss of lipid-membrane assymetry [5]. By flow cytometry

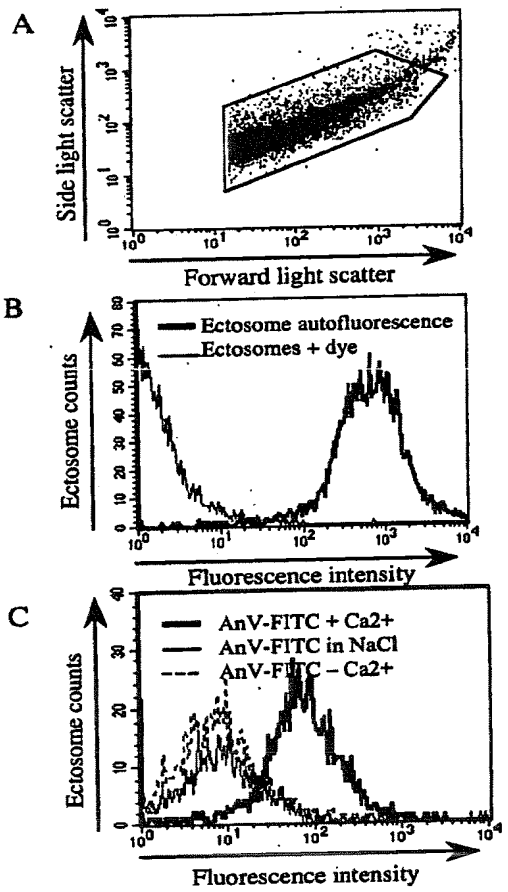


Fig. 2. Detection of ectosomes by FACScan, (A) Scatter dot plot of ectosomes derived from activated normal PMNs. The gate (R1) contains the majority of ectosomes. (B) Fluorescence histogram of ectosomes before (thin line) and after (thick line) membrane labeling. (C) Fluorescence histogram of AnV-FITC stained ectosomes. Ectosomes were incubated with AnV in binding buffer (thick line), calcium depleted binding buffer (dotted line), or 0.9% NaCl solution (thin line).

we could show that annexin V (AnV), which binds PS in a calcium-dependent manner, bound to the vesicles (Fig. 2C). This binding was prevented by depleting calcium from the buffer or using a saline solution deprived of calcium. Thus, the vesicles exposed negatively charged phospholipids, very likely to be phosphatidylserine. An AnV/membrane-dye double-labeling indicated clearly that AnV binding was present on all lipid-expressing vesicles, with the binding of AnV correlating directly with the size of the vesicles.

Protein expression by PMN-derived vesicles

We then set out to analyse the proteins expressed by these vesicles by two-dimensional gel-electrophoresis (proteome) (Fig. 3A). We identified actin and annexin III as two of the major proteins present by comparing 2-DE patterns of vesicles with a 2-DE library (computer-aided) and by sequencing (Fig. 3A and B). Actin is a cytosolic protein, which would confirm that the vesicles form by budding from the cell membrane and incorporate some cytoskeletal components of the cytosol. Flow-cytometry phalloidin, which binds F-actin, was used to confirm the presence of actin in the vesicles. Interestingly, phalloidin did react with permeabilised vesicles only, and the intensity of staining was proportional to the concentration of the detergent used (Fig. 3C). By contrast, intact vesicles did not bind phalloidin. Thus, actin was only present inside the vesicles, suggesting that they were rightside-out oriented. Permeabilisation did not alter the binding of mAb specific for proteins present on the surface of ectosomes (described below), thereby ruling out the possibility that the detection of actin was due to phalloidin trapped inside ectosomes.

In parallel, we analysed the expression of proteins on the surface of the vesicles by using mAb against different PMN proteins using purified PMNs as controls. With this approach, many transmembrane [HLA-I, CD35 (CR1), CD11a, CD11b, CD62L (L-selectin), CD46 (MCP)] and GPI-anchored proteins [DAF, CD59, CD66b, CD16 (FcγRIII)] were identified on the vesicles (Table 1). Of particular interest was the presence of L-selectin, which is cleaved off PMNs at the time of cell activation, but which is clearly lost from PMNs by vesiculation as well. In addition to proteins derived from the PMN cell surface, vesicles also expressed elastase, proteinase 3 (PR3), MPO, and MMP-9, all stored within granules of resting PMNs (Table 2), with some enzymes producing a more intense staining than others. Furthermore, by double-labeling we could confirm that the fluorescence for MPO was directly proportional to the fluorescence obtained after membrane staining (amphiphilic dye; Fig. 4C). Electronic compensation during acquisition of double-staining data was used to reduce the spectral overlap of both fluorochromes. An identical proportional increase was seen when testing CD46 (MCP) vs. AnV (Fig. 4B). Other double-labeling studies suggested that this was a general rule for ectosomal proteins, i.e., the intensity of fluorescence being proportional to membrane staining. Indeed, we tested both CD16 (FcγRIII) and elastase vs. AnV and obtained similar results (data not shown). In fact, every double-labeling suggested the same general rule: all staining intensities correlated positively, even when two membrane proteins were plotted versus each other (e.g., CR1/CD66b, Fig. 4A). In addition, the correlation between signal intensities of detected proteins and/or membrane confirmed that the detection of protein was limited by the size of the vesicles. Even the smallest vesicles were shifted by the specific label, although this shift was sometimes minimal (membrane dye single-positive, e.g., Fig. 4C, right plot, lower right-hand side quadrant). Therefore, large vesicles are likely to be more biologically active.

Furthermore, we could not detect different populations of vesicles, i.e., we found no discrepancies between all the markers used. By contrast, the vesicles (even when gating

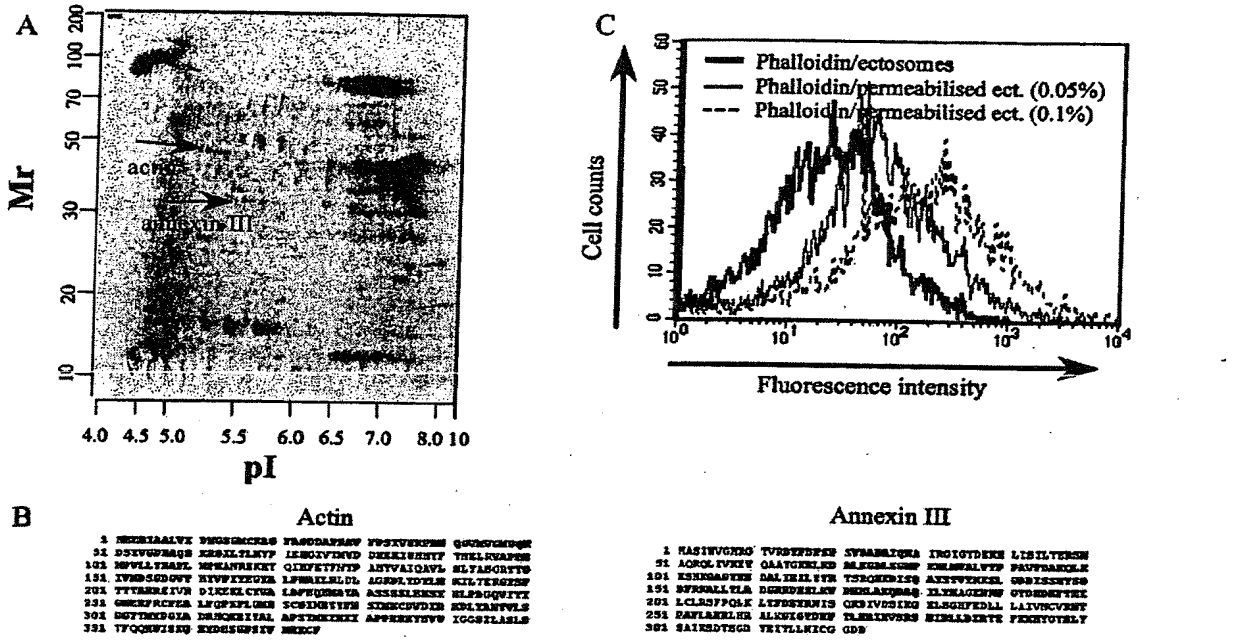
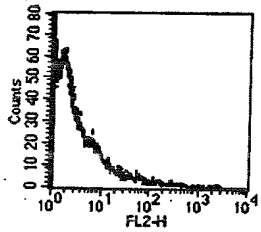
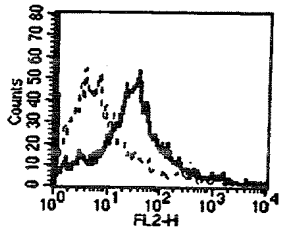


Fig. 3. Detection of ectosome-associated annexin III and intraectosomal actin. (A) 2D-GE of ectosomal proteins. By mass spectrometry, protein spots indicated by arrows were identified as actin and annexin III, as indicated. (B) Trypsin-digested excised spots were analyzed by tandem mass spectrometry and ion spectra interpreted with the Mascot database search. Peptide sequences matching the database sequence are shown in bold letters. For actin and annexin III, 21 and 16 matching peptides were obtained with sequence recovery percentages of 69% and 52%, respectively. (C) Binding of phalloidin to ectosomes was analyzed in permeabilizing buffers containing increasing concentrations of detergent (thin line: 0.05% Nonidet P40, dotted line 0.1% Nonidet P40). The control experiment was done using the same buffer without detergent (thick line).

Table 1
Summary of PMN proteins found on ectosomes by FACScan analysis

PMN proteins not detected on ectosomes	PMN proteins detected on ectosomes
Example shown[a]: uPAR/CD87	Example shown[a]: HLA-1
	
Other proteins not detected: Receptors: CD14 and FcγRIII/CD32	Other proteins detected: Receptors: CR1/CD35, LFA-1/CD11a, Mac-1/CD11b, FcγRIII/CD16, and L-selectin Enzymes: Myeloperoxidase, Elastase, MMP-9, and PR3 Complement proteins: MCP/CD46 and CD59 Other: CD66b

[a] Dotted line represents fluorescences of ectosomes (in R1) incubated with isotype-control mAb; thick line represents fluorescences of ectosomes (in R1) incubated with mAb against the mentioned antigen.

on their larger fraction only) did not express detectable amounts of CD14, CD32 (FcγRII), and CD87 (urokinase plasminogen activator receptor, uPAR), while the same molecules could clearly be detected on PMNs (data not shown).

Taken together, the data indicated that the vesicles have all the hallmarks of ectosomes as described by Stein and Luzio [9]. However, one additional aspect was uncovered in the sense that ectosomes may acquire enzymes, which are released by degranulation of PMNs at the time of their activation.

Binding of MPO to ectosomes

Taking advantage of an MPO-deficient individual, the mechanism by which MPO (stored within the azurophilic granules of resting PMNs) may become translocated to the membrane of ectosomes was studied. First, purified MPO in normal buffer (PBS) bound to MPO-deficient ectosomes in a dose-dependent and saturating manner (data not shown). The fluorescence intensity reflecting the presence of MPO on ectosomes reached the level of normal ectosomes (Fig. 5A and B). Second, using a more physiological assay, MPO from ectosome-free S/N from degranulated PMNs of normal donors (containing soluble MPO) bound to a similar extent to MPO-deficient ectosomes (Fig. 5B), indicating

Table 2
Cellular localisation of proteins analysed for their presence on ectosomes

	Ectosomes	Cell membrane	Azurophilic granules	Specific granules	Gelatinase granules	Secretory vesicles	Protein type
HLA-1	+[a]	+	–[b]	–	–	–	TM[c]
CR1/CD35	+	+	–	–	–	+	TM
CD11a	+	+	–	–	–	–	TM
CD11b	+	+	–	+	+	+	TM
L-selectin	+	+	–	–	–	–	TM
MCP/CD46	+	+	–	–	–	–	TM
CD66b	+	+	–	+	–	–	GPI[d]
DAF/CD55	+	+	–	–	–	–	GPI
FcγRIII/CD16	+	+	–	–	–	+	GPI
CD59	+	+	–	–	–	–	GPI
uPAR/CD87	–	+	–	+	+	+	GPI
CD14	–	+	–	–	–	+	GPI
FcγRII/CD32	–	+	–	–	–	–	TM
MPO	+	–	+	–	–	–	Soluble
Elastase	+	–	+	–	–	–	Soluble
Proteinase 3	+	–	+	+	–	+	Soluble
MMP-9	+	–	–	+	+	–	Soluble

[a] Present.
[b] Absent.
[c] Transmembrane-anchored protein.
[d] Glycosyl-phosphatidylinositol linked protein.

that at least a fraction of MPO rebinds to the surface of the ectosomes.

Functional analysis of ectosomal enzymes

The next question was to see whether the enzymes fixed on ectosomes remained functionally active. In an assay for MPO-mediated catabolism of hydrogen peroxide, whole ectosomes were found to be capable of oxidizing orthophenylenediamine (Fig. 5C). This also held true for ectosomes that acquired MPO from the soluble phase (data not shown).

The presence of matrix metalloproteinase-9 (MMP-9) was confirmed by Western blot of ectosomal proteins (Fig. 5D, upper panel). The migration of the monomeric form of MMP-9 from ectosomes was slightly different from the control (active) MMP-9, suggesting that it was in its proform. We obtained three distinct bands of apparent molecular weights 92, 130, and 220 kDa, which seem to correspond to all three storage-forms of MMP-9 in PMNs, namely pro-MMP-9 monomer, the MMP-9/lipocalin heterodimer, and the MMP-9 homodimer, respectively. All forms of MMP-9 were then shown to be enzymatically active by their capacity to degrade gelatin in a zymographic assay (Fig. 5D, lower panel; only monomeric MMP-9 shown).

Whole ectosomes had elastolytic activity, which was in part inhibitable by elafin, a specific inhibitor of elastase/PR3 (Fig. 5C). Elastase as well as MMP-9 activities remained unchanged when ectosomes were first lysed by sonication (data not shown). These data suggest that elastase/PR3 and MMP-9 were present almost exclusively on the surface of ectosomes and not stored inside ectosomes.

Ectosomes released by PMNs can be identified in whole blood

An important aspect was to see whether the reactions observed with purified PMNs would be similar in a more physiological environment, and would correspond to the activation of circulating PMNs as described in several clinical situations [26–28]. Therefore, PMN activation in whole blood was simulated in vitro, and a system to specifically trace PMN-derived ectosomes was established. fMLP was added to citrate-anticoagulated blood and incubated for 20 min at 37°C. Thereafter, cells were removed and the S/N applied to ultracentrifugation. The pellet was redissolved and analysed by FACScan. A typical FSC/SSC plot of microparticles obtained from activated whole blood is shown in Fig. 6A. Phosphatidylserine expressing membrane particles were traced by staining with AnV, and PMN-derived ectosomes were distinguished from non-PMN-derived microparticles by tracing the PMN-specific protein CD66b. As shown in Fig. 6B, a majority of particles obtained from the S/N of activated whole blood was binding AnV (≈70%). In contrast, only a subpopulation of AnV-binding particles also expressed CD66b (≈13% of the total events or ≈20% of AnV positive events; Fig. 6C). The AnV/

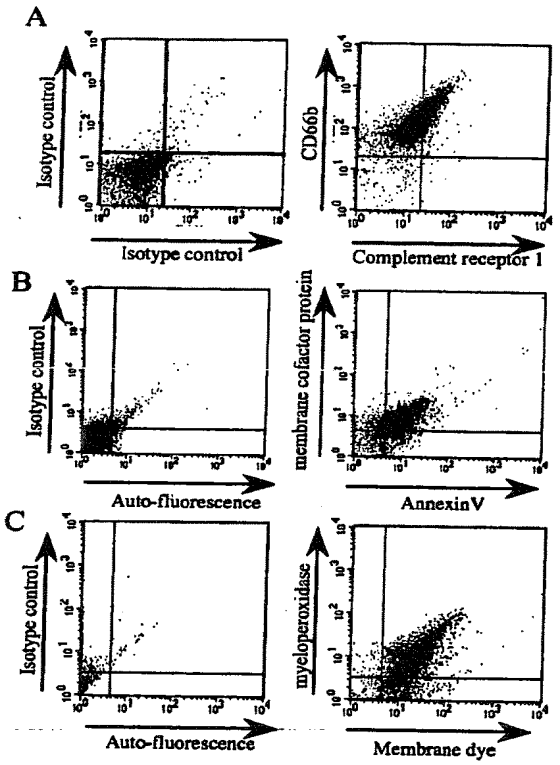


Fig. 4. FACScan analysis of protein/protein and membrane/protein double-labeled ectosomes. (A) Isotype-control double-labeled ectosomes are shown on the left, CD35/CD66b double-labeled ectosomes on the right. (B) Isotype-control labeled ectosomes are shown on the left, AnV/CD46 double-labeled ectosomes on the right. (C) Isotype-control labeled ectosomes are shown on the left, membrane dye/MPO double-labeled ectosomes on the right. All data represent fluorescence intensities of ectosomes gated in R1.

CD66b-double-positive population (i.e., PMN-derived ectosomes) localised in a distinct region of the FSC/SSC plot (Fig. 6C), comparable to the one delimited by ectosomes obtained from isolated PMNs (Fig. 2A). This gate was devoid of CD66b negative, i.e., non-PMN-derived particles, indicating that PMN-derived ectosomes exhibit particular biophysical characteristics distinguishing them from other blood-borne microparticles by FACScan. CD66b-positive (PMN-derived), as well as single-, AnV-positive ectosomes could be detected using the same technique in control plasma (without fMLP), however, in significantly lower numbers than in the supernatant of blood stimulated with fMLP (data not shown). This finding is consistent with previous reports describing the presence of ectosomes in the circulation of healthy individuals [19,29].

Ectosomes bind to the surface of selective cell types

Because ectosomes express various adhesion proteins including L-selectin, we tested for their capacity to bind to endothelial cells (HUVEC) and a human monocytic cell line (THP-1) using human erythrocytes as controls. Ectosomes were labeled with membrane dye and incubated with the aforementioned cell types. Thereafter, cells were washed extensively in order to release weakly associated ectosomes. As assessed by FACScan analysis, both HUVEC and THP-1 cells bound ectosomes in a dose-dependent, and saturable manner. Competition for binding-sites on HUVEC and THP-1 cells was revealed by their displacement with unlabeled ectosomes (Fig. 7A and B), a displacement which was concentration dependent. No binding of ectosomes to erythrocytes was observed (Fig. 7E). This was in contrast to liposomes [70% PS, 30 % phosphatidylcholine (PC)], which were found to bind nonspecifically to the cell surface of all three cell types (data not shown). Using confocal microscopy, fluorescent ectosomes were visualised as discrete spots on the cell surface of both HUVEC and THP-1 cells, confirming the data obtained by FACScan (Fig. 7C and D).

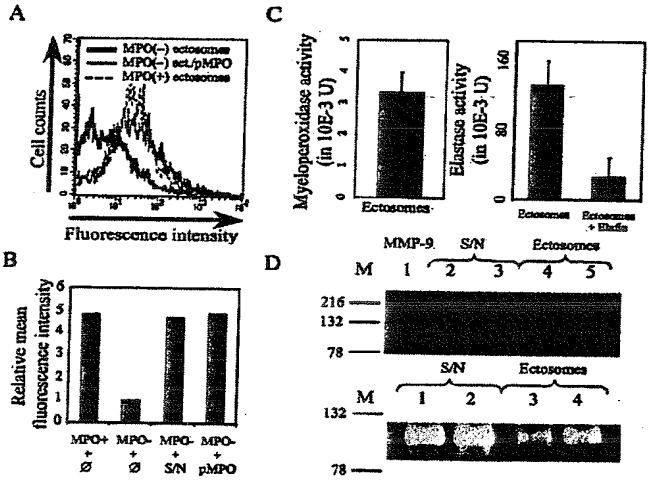


Fig. 5. Ectosomes expose active MPO, PR3, and MMP-9 at their surface. (A) FACScan-analysis of MPO expression on ectosomes derived from a normal donor (dotted line) and an MPO-deficient individual incubated with purified MPO (thin line) or without MPO (thick line) (gate R1). (B) Relative mean fluorescence intensities of ectosomes derived from a normal donor (column 1) and an MPO-deficient donor (column 2). Ectosomes derived from an MPO-deficient individual were incubated with MPO-containing, ectosome-free S/N from normal degranulated PMNs (column 3), or with purified MPO (column 4). The arbitrary value 1 was set for MPO-deficient ectosomes. (C) Left hand panel, ectosome-associated MPO activity expressed in units (U) released by 10^6 activated PMNs within 20 min. Data represent results from two independent experiments. Right hand panel, ectosome-associated elastase activity in presence (column 1)/absence (column 2) of elafin. Activity is expressed in U released by 10^6 activated PMNs within 20 min, data represent results from four independent experiments for each assay. (D) Western blot analysis of ectosomes, ectosome-free S/N, and rMMP-9. Each lane corresponds to an independently prepared sample. MMP-9 activity of ectosomes and ectosome-free S/N as assessed by gelatin zymography. Each lane represents an independently prepared sample.

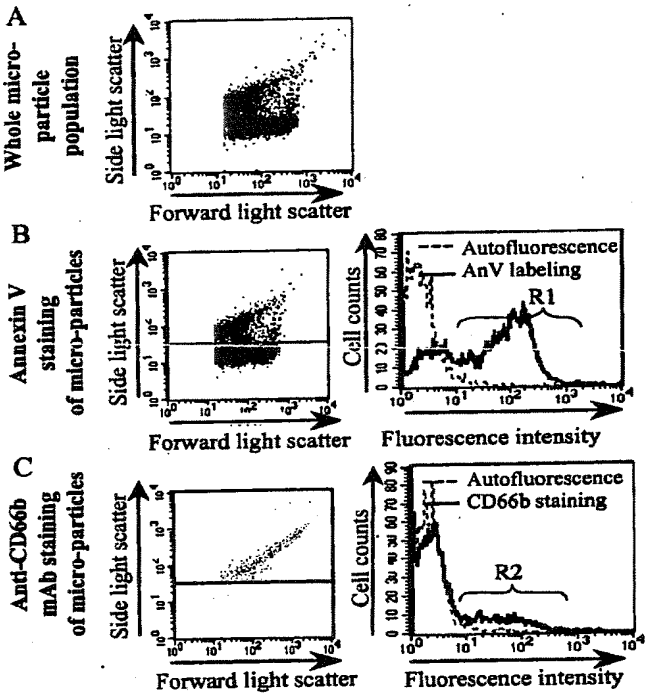


Fig. 6. Detection of ectosomes in whole blood. (A) Scatter dot plot of total microparticles (MP) derived from fMLP stimulated whole blood. (B) The dotted and filled lines in the histogram correspond to unstained and AnV-FITC stained blood MP, respectively. Gate R1 includes all AnV-positive MP. The scatter dot plot represents FSC/SSC characteristics of AnV-positive MP only (gated on R1). (C) The dotted and filled lines in the histogram correspond to unstained and anti-CD66b stained MP, respectively. Gate R2 includes all CD66b-positive MP. The scatter dot plot represents FSC/SSC characteristics of CD66b positive MP only (i.e., ectosomes, gate R2).

Binding of C1q to the surface of ectosomes

The binding of C1q to apoptotic cells/bodies has been shown to be one of the central reactions which allows the efficient removal of apoptotic cells by phagocytes. To see whether C1q would also bind to ectosomes, we incubated them for 30 min at 4°C with or without purified C1q (10 μg/ml final concentration) in 0.9% NaCl, normal human serum or heat-inactivated normal human serum (1/10 final dilutions). C1q was detected on ectosomes after incubation with purified C1q and more interestingly after incubation with whole serum, in which C1q is associated in the macromolecular C1 complex (Fig. 8). No signal was detected in the control heat inactivated serum or in the absence of C1q.

Discussion

In the present studies, we analysed biochemical and immunological characteristics of vesicles released by activated PMNs. The main findings were that these vesicles corresponded in many ways to ectosomes as described by Stein et al., because they expressed a selected set of membrane proteins, and were rightside-out oriented vesicles containing cytoplasmic proteins [9]. They had, however, a series of properties not described yet for PMN ectosomes, i.e., they expressed phosphatidylserine in the outer leaflet of their membrane similarly to apoptotic bodies and to vesicles released by platelets, they acquired enzymes originating from azurophilic granules on their membrane, and they targeted endothelial cells as well as monocytic THP-1 cells.

Using electron microscopy we have previously shown that the vesicles shed by PMNs had a size of approximately 50 to 200 nm [10]. Here, the vesicles were analysed by FACScan, which allowed us to further define them. A distinct population of membrane particles was clearly identified and when compared to scattering characteristics of synthetic beads (diameter 200 nm and bigger), these membrane particles/vesicles had the same wide range of sizes as seen on the electron microscopy images [10]. All the events registered in the defined FACScan window incorporated a lipid marker for membranes indicating that no artifacts such as aggregates of proteins were present. The intensity of labeling with this marker was directly correlated with the size of the vesicles confirming that the lipid membrane increased together with the size of the vesicle as estimated from the scattering data. Interestingly all positive protein and lipid markers used showed the same increase in fluorescent intensity with the increase in size, suggesting that vesicles may differ in size but not in their general membrane composition. Thus, activated PMNs shed one type of vesicles, which might, however, not have identical properties because of their size.

Ectosomes should contain cytosolic proteins. The presence of actin within and not on the surface of PMN-derived vesicles confirmed that they are released as intact rightside-out membrane vesicles with a cytosolic content. Actin has been shown to be associated with vesicles released by mechanically stressed fibroblasts, where it is thought to be involved in vesicle budding [13]. Actin also has been proposed to play a role in specific protein retention during in vitro budding of erythrocyte vesicles [30]. It is likely that actin, together with other cytoskeletal components, plays a role in the formation of PMN-derived ectosomes as well.

Stimulatory agents induce the shedding of membrane vesicles mainly by inducing first an influx of calcium into the cell, which is followed by the scrambling of lipids between the two layers of the cell membrane, and possibly by an active transport of phosphatidylserine [5,31,32]. These reactions result in loss of the physiological asymmetry of the membrane and the expression of phosphatidylserine on the outer leaflet. Once these events have taken place, vesicles are shed by budding from the cell membrane. We therefore expected ectosomes to express phosphatidylserine and could demonstrate it by the binding of AnV. However, normal cells can restore this asymmetry over time [14],

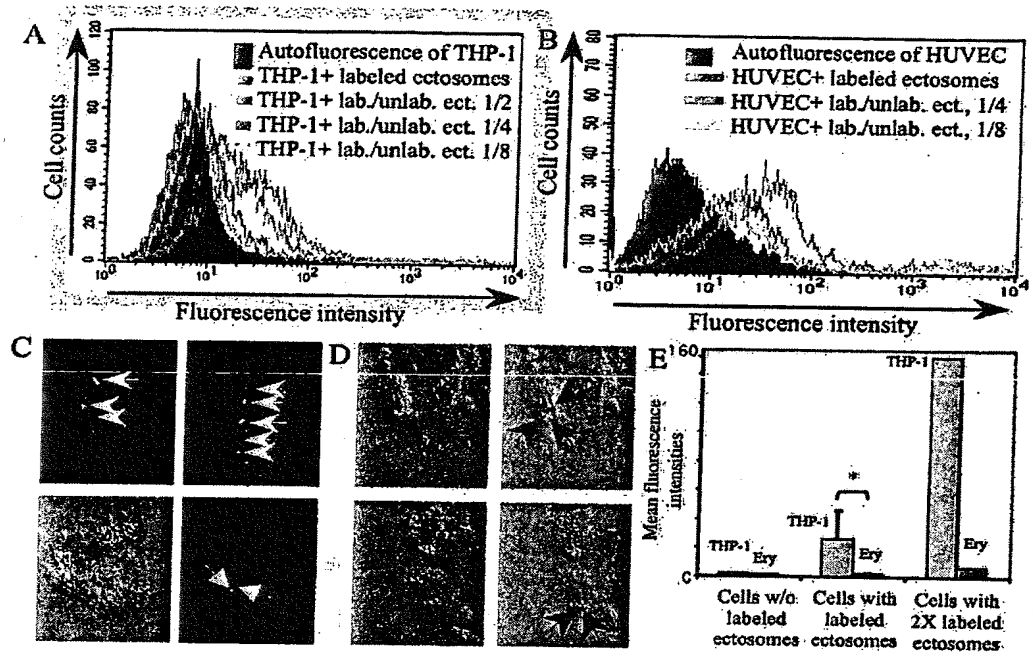


Fig. 7. Binding of ectosomes to selective cell types: THP-1 and HUVEC, but not erythrocytes. (A) Histogram plot of THP-1 cells incubated with ectosomes labeled with membrane dye. THP-1 cells were incubated with nonlabeled ectosomes prior to being incubated with labeled ectosomes. Filled violet corresponds to autofluorescence of THP-1 cells, the green line represents fluorescence of cells incubated with labeled ectosomes in absence of nonlabeled ectosomes, and the red, blue, and orange lines represent THP-1 cells incubated with nonlabeled/labeled ectosomes in a ratio of 2/1, 4/1, and 8/1, respectively. (B) Histogram plot of HUVEC incubated with fluorescent ectosomes and analysed by FACScan. HUVEC were preblocked with various amounts of nonlabeled ectosomes and subsequently incubated with a similar amount of labeled ectosomes. Filled violet corresponds to autofluorescence of HUVEC, the green line represents fluorescence of cells incubated with labeled ectosomes in absence of nonlabeled ectosomes, and blue and orange lines represent HUVEC incubated with nonlabeled/labeled ectosomes in a ratio of 4/1 and 8/1, respectively. (C) Confocal microscopy of fluorescent ectosomes bound to THP-1 cells. Upper panels, pictures of a single THP-1 cell after incubation with fluorescent ectosomes at two different focal levels. Lower panels, pictures of a THP-1 cell after incubation with fluorescent ectosomes in phase contrast (left) as well as in fluorescence modus (right). Bound fluorescent ectosomes are marked with arrows. (D) Confocal fluorescence microscopy of HUVEC after incubation with labeled ectosomes. On the left, HUVEC are shown in phase contrast; and on the right, in the fluorescence modus. Bound ectosomes are indicated with black arrows. (E) Comparison of mean fluorescence intensities (expressed in relative fluorescence units) of either THP-1 cells (left columns, THP-1) or erythrocytes (right columns, Ery) incubated 30 min without (data series 1) or with increasing amounts (data series 2–3) of labeled ectosomes (*P = 0.16 with Student's t-test). Experiments were carried out in parallel with same amounts/concentration of cells in each assay. Results are the mean ± SEM of two experiments. Data shown in all five parts of this figure were obtained with different ectosome preparations.

which was not the case for ectosomes (data not shown). This may reflect insufficient flipase activity to restore the asymmetry owing to an insufficient amount of the enzyme and/or the absence of ADP/ATP regeneration within ectosomes. Phosphatidylserine exposed on different types of microvesicles/ectosomes, including those derived from platelets, monocytes, and endothelial cells mediates procoagulant activities by recruiting coagulation factors [5,20,33–37]. Similar procoagulant properties have been attributed to PMN-derived ectosomes [38].

The second ectosomal protein identified by 2-DE/protein sequencing was annexin III. Annexin III is mainly expressed in PMNs. In resting PMNs it is associated with cytoplasmic granules, but translocates to the plasma membrane as soon as the cells get activated [39]. It is known to bind indiscriminately to membrane surface in a calcium-dependent manner. Thus, it is very likely that annexin III associates to forming ectosomes at the time of PMN activation. The physiological role(s) of this protein are hitherto still largely unknown. However, annexin III has been proposed to mediate vesicle fusion in secretory cells and might be involved in exocytosis. It might therefore play an active role in the process of ectocytosis as well.

We have previously shown by coimmunoprecipitation, that CR1-bearing ectosomes coexpress HLA-I, CD46, CD55, and CD59, while CR1-bearing ectosomes did not coimmunoprecipitate with detectable amounts of CD14 [10]. FACScan-based analysis confirmed these findings, adding evidence for a specific ectosomal protein expression, not simply corresponding to protein expression on the PMN cell-surface membrane (absence of CD14 but also of CD32 and CD87). The selection for ectosomal protein expression did not segregate between transmembrane- (TM-) and GPI-anchored proteins because both types of molecules were present (e.g., $CR1_{TM}$, $CD55_{GPI}$), respectively absent (e.g., $CD14_{TM}$, $CD32_{GPI}$) on ectosomes. However, our technique

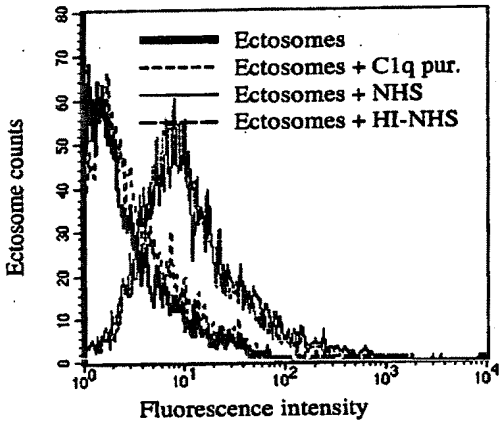


Fig. 8. Binding of C1q to ectosomes. FACScan histogram of ectosomes incubated for 30 min at 4°C in the presence or absence of C1q and subsequently probed with a biotinylated anti-C1q mAb and streptavidin-phycoerythrin. The bold and short dotted lines represent ectosomes incubated in 0.9% NaCl in the absence and presence of 10 μg/mL purified C1q, respectively. The thin and long dotted lines represent ectosomes incubated in 0.9% NaCl in the presence of 10% (final dilution) normal human serum and heat-inactivated normal human serum, respectively.

did not allow us to define whether some of the molecule were enriched in the ectosomes as compared with the PMN membrane. Such enrichment has been described on ectosomes released by erythrocytes for GPI-anchored proteins, but interestingly for CR1 as well [40]. Taken together, these data would suggest that at the time of ectocytosis, the shuffling of the membrane proteins follows rules, which are not specifically dependent on GPI anchoring. Upon activation, PMNs are known to shed very efficiently L-selectin (CD62L), as well as CD11b [41–46], CR1[47], and many other molecules [48–50]. Interestingly, ectosomes were found to express some of these molecules. Thus, early during activation, PMNs release a set of cell surface molecules by proteolytic cleavage but also by ectocytosis, indicating that both processes take place in an orchestrated manner. Shedding of cell surface molecules is thought to be a means for a rapid adaptation of the cellular phenotype [49]. For instance, shedding of L-selectin (CD62L) is known to be important during extravasation of PMNs in the transition from a rolling phenotype to firm endothelial adhesion [51,52]. In this respect, ectocytosis might be viewed as an additional efficient mechanism to modify the PMN cell surface phenotype. On the other hand, the presence of CD62L on ectosomes might be essential for their adhesion properties. This observation suggests as well that enzymatic cleavage differs on the PMN cell membrane and sheds ectosomes. Smith et al. have recently shown that the susceptibility of erythrocytes to secretory phospholipase A2 after exposure to calcium ionophore was related to the specific properties of the lipid membrane, i.e., it was proportional to the increased order of membrane lipids [53]. One might speculate that, for ectosomes as well, the enzymatic susceptibility of membrane molecules is related somehow to changes in the membrane lipid organisation.

Among the most fascinating characteristics of PMN-derived ectosomes was the fact that they selectively combined molecules well segregated in resting PMNs, i.e., a series of adhesion molecules and many but not all enzymes stored within azurophilic granules. This unique grouping was related to different processes—selection of membrane proteins for adhesion molecules and specific binding of enzymes to the outer leaflet of the ectosome membrane. How enzymes are translocated to this site and the types of binding are ill defined. Taking advantage of MPO-deficient PMNs, we studied the mechanism of MPO translocation to ectosomes. Ectosomes released from MPO-deficient PMNs were found to acquire MPO when exposed to S/N of degranulated normal PMNs (containing soluble MPO) or to purified MPO. This binding is not unique for ectosomes, because MPO is known to bind to resting PMNs as well [25]. Because the ectosomal membrane is negatively charged, electrostatic interactions with cationic MPO are very likely to contribute to the binding. In contrast, PR3, another cationic enzyme present on PMN-derived ectosomes, has recently been shown *not* to rebind to PMN membranes [25]. PR3 instead translocates to the cell surface directly, exhibiting strong (possibly covalent) interactions with the membrane. Whether a fraction of MPO becomes translocated in a similar manner cannot be excluded. Elastase, MPO, and MMP-9 were in an enzymatically active form on whole, intact ectosomes. After disrupting the ectosomes by sonication, there was no increase in the enzymatic activity of MMP-9 and elastase suggesting that this activity was limited to the outerside of the ectosome membranes with no storage compartment inside ectosomes.

In addition to being present on PMN-derived ectosomes, active MMP-9 has been demonstrated on ectosomes derived from fibrosarcoma and breast carcinoma cells, as well as endothelial cells [22,23,54]. PMNs store MMP-9 in three different pro-forms: monomers of 92 kDa, dimers of 220 kDa, and MMP-9/lipocalin heterodimers of about 130 kDa [55]. All forms are released upon PMN activation and we found all storage forms on the surface of ectosomes. Subsequent activation of MMP-9 is tightly controlled by ubiquitous tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) [56,57]. Ectosomal MMP-9 might therefore become fully active thanks to elastase, which is known to inactivate TIMP-1 [58]. Distinct ectosomal sets of molecules may locally shift the balance between inhibitory and activatory molecules, thereby allowing specific enzymes to escape inhibition. Besides acting on molecules in their microenvironment, ectosomal enzymes might also act on proteins expressed on the same ectosomes. This might explain the absence on ectosomes of certain proteins sensitive to proteolytic cleavage [e.g., CD14, uPAR (CD87)][59–61]. CD14, for instance, has been shown to be sensitive to proteolytic cleavage by cathepsin G [59]. Cathepsin G is released at the time of PMN activation; whether it is present

on the surface of ectosomes is yet unknown. However, both soluble and ectosome-bound cathepsin G might cleave off CD14 from the surface of ectosomes. The overall cleavage process would, however, be very selective, because other molecules such as L-selectin, although shed from PMNs, are not cleaved from the surface of ectosomes. Uniting adhesion molecules with antimicrobial enzymes (MPO, PR3, and/or elastase) [62–64] or matrix-degrading enzymes (elastase, PR3, and/or MMP-9) [63,65–68] may target "effector" molecules to specific locations. This might be beneficial, e.g., by focusing antimicrobial activity on opsonised bacteria, but also may contribute to local tissue destruction or cell activation.

The expression on ectosomes of negatively charged lipids (such as phosphatidylserine) as well as different adhesion molecules suggested that they might be able to specifically bind to cells. Indeed, ectosomes selectively and specifically bound HUVEC and THP-1 cells with high affinity/avidity, and not to erythrocytes. Many receptor(s) or molecule(s) of ectosomes may be involved in this binding, including L-selectin, and phosphatidylserine [69–72]. Interestingly, the addition of annexin V did not decrease or even block the binding of ectosomes to THP-1 cells (data not shown). We believe that the binding of ectosome to selective cell types might not occur through just one type of molecular interaction: blockade of one mechanism might be compensated by several others. Importantly, cytochalasin D did not prevent THP-1 cells to become fluorescent in our assays (data not shown), confirming that our experimental conditions tested binding. Longer incubation times might allow phagocytosis of ectosomes to occur. Whether phagocytosis of ectosomes induces a proinflammatory or antiinflammatory state in monocytes remains an intriguing question, particularly when one considers that ectosomes may have some similarities with apoptotic bodies which are under physiological circumstances cleared without producing harm [73–76].

C1q has been shown to bind to the surface blebs of apoptotic cells, a binding which occurs via the globular heads of C1q [77]. Recent evidence suggests that the binding of C1q is important for the nonphlogistic clearance of apoptotic cells, thus preventing the development of autoimmunity against the many newly expressed antigens on apoptotic cells. Interestingly, C1q deficiency individuals develop an autoimmune disease, which is very similar to systemic lupus erythematosus [78]. Here we showed that ectosomes bind C1q from serum similarly to apoptotic cells. We have no evidence yet to suggest that the binding and clearance of ectosomes is regulated by C1q binding, but from the foregoing, this possibility remains open.

In summary, many of the biochemical characteristics of ectosomes of PMNs have been analysed in the present work. The observations may open new avenues in the way we think about the biological role of the released ectosomes. In addition, the clear definition of ectosomes, as detailed here, could serve as a basis for a unified terminology, which would replace the multiple names used until now (microparticles, microvesicles, etc.). This would also underline the difference between ectosomes and exosomes which are formed by another process and might have very different functions.

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